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IFN-γ +875 microsatellite polymorphism as a potential protection marker for leprosy patients from Amazonas state, Brazil

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ABSTRACT

Polymorphisms present in the first intron of IFN-γ may have an important role in the regulation of the immune response, which could have functional consequences for gene transcription. Leprosy patients are characterized by different immune responses in different clinical forms. We investigated a possible association of the +874 polymorphism and CA repeats present in the first intron of IFN-γ with susceptibility to leprosy and with the manifestation of the different clinical forms. Nucleotide sequencing was performed with samples from 108 leprosy patients and 113 controls subjects, as well as immunophenotyping of CD_4^+, CD_8^+ and CD_{69}^+ T cells by flow cytometry. The data showed that there were no significant differences between patients and control subjects, as well as according classification of Ridley–Jopling. However, the A/A genotype was significantly increased in paucibacillary patients ($p = 0.028$) and the microsatellite encoding 16 CA repeats were significantly associated with paucibacillary compared to multibacillary patients ($p = 0.019$). Individuals homozygous for the +874 A allele, the mean level of CD_4^+ and CD_{69}^+ T cells was higher. Our data suggest that polymorphisms present in the first intron of IFN-γ are not associated with susceptibility to leprosy, nevertheless, the +874 polymorphism and the CA repeats number encoded in IFN-γ gene may be related to a higher cellular immune response in patients and are consistently more frequently detected in PB patients.

1. Introduction

Leprosy is an infectious disease characterized by different clinical forms that are associated with the type of immune response against *Mycobacterium leprae*. Patients with the tuberculoid form present a higher cellular immune response, while those with the lepromatous show an increased humoral immune response [1]. The cytokine IFN-γ plays an important role in the activation of NK cells and induction of Th-1 cells in the development of the host immune response [2].

The IFN-γ gene is located on chromosome 12q14.1 and consists of four exons with three intervening introns over approximately six kilobases (kb). Although few genetic polymorphisms have been characterized at this locus, some polymorphisms were described in the non-coding region [3]. To our knowledge the first report of an IFN-γ polymorphism, a CA microsatellite in the first intron of the gene that was associated with cytokine levels, was detected by Pravica et al. [4]. In their study, the authors observed higher IFN-γ expression when 2 alleles encoding 12 CA repeats were present in the genome. A second study demonstrated an absolute correlation of 12 CA repeat with the +874 T allele. However, the +874 single-nucleotide polymorphism (SNP) has also been related to IFN-γ production [5].

The +874 SNP coincides with the binding site for the transcription factor NF-κB [6], therefore, this SNP may have an important role in the regulation of immune responses by affecting NF-κB-mediated transcription of the IFN-γ gene. The NF-κB motif is ANTYYC, with N representing any nucleotide and Y representing a cytosine or thymine [7]. When the +874 T allele is present, the sequence AATCTC is close to the +874 SNP. However, the +874 T/A polymorphism can affect IFN-γ levels when the A allele is present, the binding site of NF-κB is altered [5]. This polymorphism has been associated with tuberculosis [8–10] and toxoplasmosis susceptibility [11]. Individuals from Brazil and Spain with the +874 A/A genotype express lower IFN-γ levels than those with the A/T and T/T genotypes [8,12]. In different states of Brazil, such as Minas Gerais [13], Paraná [14] and Rio de Janeiro [15], the A allele is more

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frequently found in the population. Accordingly to those data, our hypothesis is that this +874 A/A genotype is associated with the low cellular response observed in multibacillary patients. In the present study, we investigated whether the +874 T/A SNP and the microsatellite CA repeats are associated with susceptibility to leprosy or with the clinical leprosy form that develops in patients from Amazonas state, Brazil.

2. Study subjects and methods

2.1. Study population

The case-control study consisted of 108 patients with leprosy and 113 control subjects between 18 and 65 years of age. All participants were recruited at Fundação de Dermatologia e Venereologia Alfredo da Matta (FUAM), a tertiary health facility located in Manaus, Amazonas, Brazil. All included patients were born in the Brazilian Legal Amazon states. Informed consent was obtained from all participants and the study was approved by the institutional Ethical Committee.

Experienced professionals from FUAM performed the leprosy diagnosis using clinical evaluation methods, which included bacteriological and histological tests. Patients with leprosy were classified based on clinical and histological criteria according to the Ridley–Jopling classification system and were also classified as paucibacillary (PB) or as multibacillary (MB). PB leprosy is characterized by the presence of $\leqslant$5 skin lesions and a bacteriologic index (BI) of 0, the classification is further subdivided into indeterminate (I), tuberculoid (TT) or borderline tuberculoid (BT) leprosy. MB leprosy is characterized by the presence of >5 skin lesions and a BI >0, the classification can be further histologically characterized as mid-borderline (BB), borderline lepromatous (BL), or lepromatous (LL) leprosy. Pregnant women were excluded from the study, as well as patients with any diagnosed immunodeficiency.

The controls were examined clinically and consisted of genetically unrelated individuals. Individuals with any immunodeficiency, infectious disease or a family history of leprosy were not included.

2.2. Biological material

The peripheral blood of control individuals and patients was collected and used for flow cytometry and DNA extraction using the *Wizard® Genomic DNA Purification* Kit, according to manufacturer's instructions (Promega, Madison, Wisconsin). After elution, DNA was stored at −80 °C until use.

2.3. Polymerase chain reaction – PCR

Approximately 50–200 ng of DNA from each sample was used for PCR. We designed the forward primer with the sequence 5′-TCGTTGCTCACTGGGATTTTG-3′. The reverse primer used was identical to one reported by Bozzi et al. [13], with the sequence 5′-CATCTACTGTGCCTTCCTGT-3′. The amplified 322 bp products were separated by electrophoresis with a 2% agarose gel.

2.4. Purification of PCR products and nucleotide sequencing

The PCR product was purified with the *Wizard® SV* Kit (Promega, Madison, Wisconsin), and approximately 3–10 ng of DNA was used in the sequencing reaction. Sequencing was performed using *BigDye® Terminator v3.1 Cycle Sequencing* (Applied Biosystems, Foster City, California) with same primers used in the PCR. Capillary electrophoresis was performed using the ABI 3130 DNA sequencer and polymer POP-7™.

2.5. Sequence analysis

The sequences were initially analyzed using the *Sequencing Analysis* (Applied Biosystems, v5.3.1) software and were further analyzed with *SeqMan* (DNASTAR Lasergene, v7.0) software to generate contigs and for comparison with the IFN-γ reference gene sequence (GenBank ID: J00219).

2.6. Flow cytometry analysis

Flow cytometry was performed using fluorochrome-conjugated antibodies against cell surface antigens to identify the T cells as CD_4^+ T cells (CD_3^+ and CD_4^+), CD_8^+ T cells (CD_3^+ and CD_8^+) or activated T cells (CD_3^+ and CD_{69}^+) (Becton–Dickinson, California). The population of T cells was identified and gated, and 10,000 events were acquired for each sample. The samples were analyzed in FACS Calibur flow cytometer (Becton–Dickinson Immunocytometry Systems, Palo Alto, California) using *CellQuest* (v.3.1) software.

2.7. Statistical analysis

The Mann–Whitney test and the χ^2 test were used to compare the variation in age and sex between the patients and control subjects, respectively. The genotype and allele frequencies present in each population were initially determined by direct counting; χ^2 tests were then performed to compare these factors between patients and control subjects and between paucibacillary (PB) and multibacillary (MB) patients as well as to compare allele frequency between PB and MB patients. The G test was used to compare genotype frequency and the microsatellite distribution between PB and MB patients. The χ^2 test was applied to each population to investigate the Hardy–Weinberg equilibrium. The odds ratio (OR) and p values were calculated using *BioEstat* (v.5.2) software. A t test was used to compare the T-cell subsets using *GraphPad Prism* (v.5.0) software. p values less than 0.05 were considered significant.

3. Results

3.1. Study population

The participants were all born in the Brazilian Legal Amazon states and each had at least a third degree of kinship from the region. There were no differences between patient and control subjects with regard to age, but gender was skewed, with a high prevalence of male patients. The distribution of patients with PB or MB is similar, with 49.1% and 50.9%, respectively (Table 1).

3.2. Genotype and allele frequencies of the IFN-γ +874 A/T polymorphism

The distribution of genotypes in patient and control subjects is at Hardy–Weinberg equilibrium (Table 2). The IFN-γ +874 A/A and A/T genotypes were more often found than the T/T genotypes in both control and leprosy patients. Significantly increased frequencies of the IFN-γ +874 A/A genotype were noted in PB ($p = 0.028$, Table 3), as well as in patients with the TT and BT forms (data not shown). No significant differences the Ridley–Jopling classification was observed between variant genotypes.

Allele frequencies for IFN-γ +874 are given in Table 3. Allele frequencies did not differ significantly between leprosy patients (classified using Ridley–Jopling) and healthy individuals. Additionally, there were no differences observed between PB and MB patients. Strikingly, the data indicated that individuals T carriers +874 locus

Table 1
Characteristics of the study population.

	Patients (n = 108)	Controls (n = 113)
Age (years)	n (%)	n (%)
Mean	36.4 ± 13.1	36.2 ± 12.1
Gender		
Male	69 (63.9)	42 (37.2)
Female	39 (36.1)	71 (62.8)
Operational classification		
PB	53 (49.1)	
MB	55 (50.9)	
Ridley–Jopling		
TT	9 (8.3)	
BT	28 (25.9)	
BB	7 (6.5)	
BV	19 (17.6)	
VV	29 (26.8)	

Tuberculoid (TT); Borderline-Tuberculoid (BT); Mid-Borderline (BB); Borderline-Lepromatous (BL); Lepromatous (LL). Mann Whitney Test for age between patients and control subjects $p > 0.05$; χ^2 test for gender $p < 0.0001$.

Table 2
Distribution of genotypes and alleles of +874 IFN-γ in leprosy patients and control subjects.

Genotype/allele	*N* (frequency)		
	Patients	Controls	OR (95% IC)
A/A	65 (60.2)	61 (53.9)	1.29 (0.76–2.20)
A/T	33 (30.6)	43 (38.1)	0.72 (0.41–1.25)
T/T	10 (9.2)	9 (8)	1.18 (0.46–3.02)
A	163 (75.5)	147 (73)	1.28 (0.83–1.96)
T	53 (24.5)	61 (27)	0.78 (0.51–1.20)
T carriers	43 (39.8)	52 (46.1)	0.77 (0.45–1.32)

χ^2 Test with contingency table for genotype and allele between patients and control subjects, $p > 0.05$.

Table 3
Distribution of genotypes and alleles of +874 de IFN-γ between PB and MB patients.

Genotype[a]/allele[b]	*N* (frequency)		
	PB patients	MB patients	OR (95% IC)
A/A	35 (66.1)[c]	30 (54.5)[d]	1.62 (0.74–3.53)
A/T	13 (24.5)	20 (36.4)	0.57 (0.25–1.31)
T/T	5 (9.4)	5 (9.1)	1.04 (0.28–3.83)
A	83 (78.3)	80 (72.7)	1.35 (0.73–2.53)
T	23 (21.7)	30 (27.3)	0.74 (0.40–1.38)
T carriers	18 (33.9)[c]	25 (45.5)[d]	0.62 (0.28–1.34)

[a] G Test for genotype between PB and MB patients, $p = 0.089$.
[b] χ^2 Test with contingency table for allele between PB and MB patients, $p = 0.427$.
[c] χ^2 Test for A/A genotype compared T carriers in PB patients, $p = 0.028$.
[d] χ^2 Test for A/A genotype compared T carriers in MB patients, $p = 0.589$.

display a 23% resistance to leprosy when compared to the OR of leprosy patients and controls (Table 2).

3.3. IFN-γ +874 A/T polymorphism and T-cell subsets

We observed a similar distribution of CD_8^+ T cells between genotypes (data not shown); however, a higher median of CD_4^+ T cells (Fig. 1A), as well as activated T cells (Fig. 1B) was found in individuals homozygous for the +874 A allele.

3.4. +875 CA repeats

Five alleles were identified with 12–16 CA repeats after the +874 polymorphism. A larger number of encoded CA repeats was associated with an increased risk of leprosy development, as indicated by the OR data (Table 4).

Allele 2 is found more frequently in control subjects than in patients (Table 4); however, no significant difference was observed. Significant differences were not observed between the clinical forms of leprosy, but allele 6 (encoding 16 repeats) was associated with PB patients (Table 5).

4. Discussion

IFN-γ is an important cytokine that mediates activation of innate immune cells and the development of an adaptive immune response [2]. It has been observed that a robust cellular response can lead to nerve damage in leprosy patients [16]. Previous studies have associated polymorphisms present in the first intron of the IFN-γ gene with cytokine levels in healthy subjects [4,5] and tuberculosis patients [8,12].

Some genetic polymorphisms have different outcomes in ethnically distinct populations [15,17–18]. All participants included in this study were diligently confirmed to be native to location studied, thus avoiding a genetically heterogeneous population. Age can be directly related to the development of leprosy, as older individuals have a higher risk of developing the disease [19]. To avoid this complication, the populations examined in this study were of a similar age (Table 1).

We observed a higher frequency of the +874 A/A and A/T genotypes, both in patients and control subjects, similar to what has been reported in other studies, including England [5], China [20], Japan [21], Brazil [22] and India [23], indicating similarities on different continents. In Brazil, the allelic frequency among patients is in agreement with previous studies performed in Minas Gerais [13], Paraná [14] and Rio de Janeiro [15]; thus the A allele appears to be common in the Brazilian population and may be related to the high leprosy rates in Brazil. Other studies found that the +874 A/A genotype was present at a higher frequency in tuberculosis patients, whereas heterozygous genotype was more frequent in controls [8,9,12]. Accordingly, the A/A genotype was most frequently found among leprosy patients and the A/T genotype was most frequently found in control subjects (Table 2).

The +874 A/T polymorphism was associated with tuberculosis or disease severity [8,9,12,20,24], in previous studies, individuals homozygous for the A allele had double the likelihood of developing tuberculosis compared to heterozygotes [12,20]. Heterozygosity may play an important role in the transmission of tuberculosis, because is found in a higher proportion of the healthy and tuberculin-positive contacts of tuberculosis patients [12]. Our data indicated that the individuals +874 T carriers demonstrate a 23% resistance to leprosy, which may suggest that this polymorphism plays an important role in the innate immune response.

The expression level of genes involved in the innate immune response, including the toll-like receptor (TLR), nucleotide oligomerization domain receptor 2 (NOD2) and mannose receptor 1 (MRC1), can affect leprosy susceptibility [25]. The polymorphism present in the first intron of IFN-γ may is according with these genes. Thus, based on our findings and on previous studies, the +874 A/A genotype may alter the expression of genes involved in the innate immune and affect an individual's susceptibility to *M. leprae* because the A/A genotype is present more prevalent in patients than in control individuals. However, our data does not indicate an association between this genotype and the susceptibility to leprosy.

Consistently, healthy subjects and tuberculosis patients homozygous for the A allele possess CD_4^+ T and CD_8^+ T cells producing higher levels of IFN-γ [23]. Curiously, MB patients and control subjects showed a similar frequency of genotypes; nevertheless, the

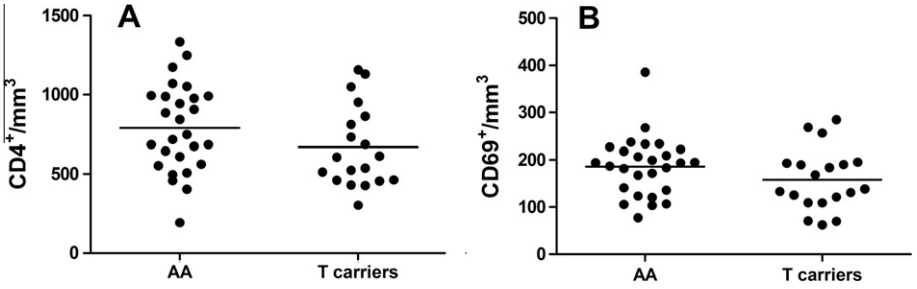

Fig. 1. CD_4^+ and CD_{69}^+ expressed in T lymphocytes from leprosy patients with AA or T carriers for +874 polymorphism. There were no significant differences in the distribution of CD_4^+ and CD_{69}^+ T cells in subjects with A/A genotype ($n = 27$) or T carriers ($n = 19$). $p > 0.05$ using t test.

Table 4
Distribution of +875 CA repeats allele between patients and control subjects.

Allele	Repeats	*N* (frequency)		
		Patients	Controls	OR (IC 95%)
2	12	40 (36.4)	52 (46)	0.67 (0.39–1.15)
3	13	2 (1.8)	3 (2.6)	0.68 (0.11–4.14)
4	14	53 (48.2)	47 (41.6)	1.31 (0.77–2.22)
5	15	2 (1.8)	2 (1.8)	1.03(0.14–7.43)
6	16	13 (11.8)	9 (8)	1.76 (0.70–4.43)

G Test for CA repeats allele between patients and control subjects, $p = 0.589$.

Table 5
Distribution of +875 CA repeats allele between paucibacillary (PB) and multibacillary (MB) patients.

Allele	Repeats	*N* (frequency)		
		PB patients	MB patients	OR (IC 95%)
2	12	17 (32.1)	23 (43.4)	0.62 (0.28–1.36)
3	13	0 (0)	2 (3.8)	–
4	14	25 (47.2)	24 (45.2)	1.08 (0.50–2.32)
5	15	0 (0)	2 (3.8)	–
6	16	11 (20.7)	2 (3.8)	6.68 (1.40–31.81)

G Test for CA repeats allele between PB and MB patients, $p = 0.019$.

frequency of the A/A genotype was increased in PB patients indicating that this polymorphism could be related to a large cellular immune response.

A previous study observed different effects of 5′ *IL12RB2* polymorphisms on NK and T cells. A certain combination of SNPs has been related to increase expression of IL12Rβ2 in T cells, while in NK cells, expression was significantly decreased. Possible explanations for these different outcomes include different binding sites for transcriptional factors in NK compared to T cells [26] and the expression of specific and different transcription factors in each cell type. Our data may in agreement with this finding, considering that the A/A genotype may be associated with a higher expression of IFN-γ in T cells but a decrease in NK cells. The +874 A/A genotype plays a role in the control of the spread of bacilli; however, this genotype provides no protect from *M. leprae* infection.

In previous studies, the +874 A/A genotype and the microsatellite repeat number have been associated with susceptibility to tuberculosis [20,27]; therefore, is hypothesized that this combination has differential effects on innate and adaptive immunity. The +874 polymorphism is associated with a functional motif, and the A/T and T/T genotypes are associated with higher expression levels of the cytokine in plasma, demonstrating that the cytokine's levels are associated with the number of copies of the T allele [8]. Therefore, individuals homozygous for the A allele have lower IFN-γ levels than those with the A/T and T/T genotypes [12]. Patients with tuberculosis have lower IFN-γ levels than healthy individuals independent of this genotype [8,28]. The +874 A/A genotype and 16 repeats of the microsatellite may be associated with a large cellular immune response.

The +874 A/A genotype may be involved in the control of infection by *M. leprae*, due this genotype was found more frequently in patients with TT and BT clinical forms (data not shown). Higher IFN-γ production has been observed in subjects homozygous for the A allele in response to the *M. leprae* [15]. This observation is confirmed in this study; we observed IFN-γ levels above 10 pg/mL in patients with the A/A genotype, whereas T carriers patients had a mean level of 4 pg/mL (data not shown). Higher IFN-γ production may be related to the number of CD_4^+ T cells and activated T cells (Fig. 1).

Previous studies have shown that allele 2 with 12 CA repeats in the first intron of the IFN-γ gene, is associated with higher levels of cytokine production *in vitro* [4]. The allele with 11 CA repeats is present at a low frequency in the general population [4,20]; recently, this finding has been corroborated [27]. This allele was not found in this study and may be considered a rare allele. The +874 T allele is correlated with the microsatellite allele 2 with 12 CA repeats [5] and we suggest that the alleles +874 A and 16 CA repeats may be associated with higher IFN-γ production and may have an important role in controlling the spread of *M. leprae*.

The functional significance of the +874 polymorphism has been observed in different tuberculosis studies, where the T allele is related to increased cytokine levels [4,5,12]. Though there is some controversy, the A/A genotype has been observed to lead to higher IFN-γ levels, specifically in response to *M. leprae* [15]. In conclusion, our results suggest that the +874 polymorphism is not associated with susceptibility to leprosy; however, the combination of +874 A/A and 16 CA repeats may be associated with a large cellular immune response to *M. leprae*, based on the higher frequency detected in PB patients.

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